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# Morphologic and Genetic Verification That Monterey *Botryllus* and Woods Hole *Botryllus* are the Same Species

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**Abstract.** To determine whether Monterey *Botryllus* and Woods Hole *Botryllus* are the same species, comparisons were made of their morphology, biology, and colony specificity. In addition, matings were carried out to ascertain whether fertile [Monterey × Woods Hole] $F_1$ progeny could be obtained. The morphology and biology of *Botryllus* colonies from Monterey and from Woods Hole are very similar, and fertile $F_1$ progeny were obtained from interpopulation crosses. Therefore, we conclude that Monterey and Woods Hole *Botryllus* belong to the same species. However, slight differences were observed in the allorecognition reactions (colony specificity) of these two populations. Although there are some inconsistencies among the descriptions of *Botryllus schlosseri* and further extensive studies of *Botryllus* taxonomy are needed, our data indicate that *Botryllus* from Monterey and from Woods Hole may be designated contingently as *B. schlosseri*.

## Introduction

Animals of the colonial ascidian genus *Botryllus* are used in scientific research in several different locations around the world, including Japan (Saito and Watanabe, 1982; Taneda and Watanabe, 1982), Italy (Sabbadin, 1977), and the United States (Grosberg, 1981; Scofield *et al.*, 1982; Mackie and Singla, 1983). On the east coast of the United States, *Botryllus* studies have been carried out at Woods Hole, Massachusetts (Milkman, 1967; Scofield and Nagashima, 1983; Grosberg and Quinn, 1986), and on the west coast in Monterey, California (Schlumpberger *et al.*, 1984; Boyd *et al.*, 1986), while some work includes data for *Botryllus* populations from both locations (Scofield *et al.*, 1982). Until now it had not been determined whether *Botryllus* from Monterey and Woods Hole are the same species. This is an important question, especially because of the tendency to apply information and conclusions obtained from experiments on one of these populations to research on the other population.

Woods Hole *Botryllus* has been called *Botryllus schlosseri* (Pallas, 1766) since Bancroft (1903) reported that "*Botryllus* at Woods Hole and at Newport exactly resembles *Botryllus* at Naples." Although this species has been regarded as cosmopolitan, inconsistencies in the literature suggest that *B. schlosseri*, as described thus far, may comprise more than one species. For example, the reported haploid number of chromosomes in the Woods Hole population (seven or eight: Milkman and Therrien, 1965) is half that of the Italian population (16: Colombera, 1969). As noted already (Van Name, 1945; Saito *et al.*, 1981a, b; Saito and Watanabe, 1985), the morphological characteristics of botryllid ascidian adult colonies and blastozooids are very similar from species to species. Therefore, detailed observations of morphology and biology throughout the life cycle are indispensable for the precise identification of the ascidians belonging to the Botryllidae. However, most of the past taxonomic reports on *B. schlosseri* dealt only with the morphological characteristics of adult colonies and blastozooids,

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whereas the morphology of eggs, embryos, larvae, oozooids, and the blastozooids of very young colonies (as well as other biological characteristics, such as colony specificity) were not included (Van Name, 1945; Tokioka, 1953). These omissions may have contributed to the confusion about the taxonomy of this species. In this study we have identified Monterey and Woods Hole *Botryllus* as members of the same species, and have also described as much about their biology and morphology as possible for future taxonomic studies of *B. schlosseri* and its possible allies.

We used two criteria that define a species in order to ascertain whether Monterey and Woods Hole *Botryllus* belong to the same species (Friday and Ingram, 1985). First, morphological characteristics of individuals from both locations were compared throughout their life cycles, because individuals within a species are more similar to one another than they are to members of other species. Second, we tested the rule that members of the same species can interbreed to produce fertile offspring. We did this by setting up defined crosses between Monterey and Woods Hole colonies in the laboratory, followed by crosses involving their $F_1$ progeny. We also compared the phenomenon and morphology of colony specificity, a kind of allorecognition manifested by fusion or nonfusion between colonies.

## Materials and Methods

### *Animals*

For the observations on morphology, both wild and laboratory-cultured colonies of Monterey and Woods Hole *Botryllus* were used. For the experiments of colony specificity and defined crosses, laboratory-cultured colonies from both populations were used. Monterey *Botryllus* colonies were raised in the laboratory from embryos released or dissected from wild colonies that had been collected from Monterey Marina (Monterey, California). Woods Hole *Botryllus* colonies were raised from embryos dissected from adult colonies that had been collected from the Eel Pond in Woods Hole, or from the Green Pond in Falmouth, Massachusetts, and shipped to our laboratory by the Marine Resources Department of the Marine Biological Laboratory (Woods Hole, Massachusetts). Colonies derived from laboratory intrapopulation crosses (*e.g.*, Woods Hole × Woods Hole) were also used. In these experiments, Woods Hole colonies were held in seawater tanks at 18°C, and Monterey and progeny colonies were held at 20°C. Details of procedures for obtaining larvae and laboratory conditions for raising colonies to sexual maturity have been previously described (Boyd *et al.*, 1986).

### *Observations on morphology*

Living colonies and fixed colonies were observed under a binocular stereomicroscope. For fixation, living colonies were immersed in 0.32 *M* $MgCl_2$ for about 15 min to anesthetize them, and then were transferred to 10% formalin in filtered seawater.

### *Colony specificity*

Colony specificity was examined between colonies of the same population and between colonies of the two different populations by means of the "cut colony assay" (Oka and Watanabe, 1957). A single system (about 15 zooids) was dissected from each of two colonies. The two systems were placed in juxtaposition on a glass slide to allow contact with each other at their growing edges. After incubation for 30–40 min in a moisture chamber, the slide was transferred to a laboratory seawater tank. Observations of the colony specificity reaction were made using a binocular stereomicroscope. The timing and details of tunic fusion, ampullar fusion or deterioration, and blood cell infiltration were recorded as the two colonies underwent fusion or nonfusion.

### *Defined crosses*

To use a genetic marker for verification of successful cross-fertilization in Monterey × Woods Hole crosses, we chose Monterey colonies lacking intersiphonal double bands and Woods Hole colonies having double bands. The parental colonies of a cross were placed in a 4-liter 20°C seawater tank at a time when their sexual stages were appropriately matched so that the sperm of one colony would be ripe when the eggs of its partner were ready to be fertilized. If necessary, the two colonies were held at different temperatures to adjust their reproductive stages prior to the cross.

Periodically during the cross, one or two zooids of each parental colony were dissected to determine the developmental stage of the embryos. Upon maturation, the tadpoles swam out of the maternal colony and attached to a glass settlement slide, where they metamorphosed to become oozooids. Because the *Botryllus* life cycle permits the colonies of a mating pair to alternate as egg and sperm donor for each other, several reciprocal fertilizations and subsequent hatches occurred for each colony pair. The progeny were kept in 4-liter 15°C tanks for two to three weeks, and then were placed in 17-liter 20°C tanks. A subset of the oozooids from each cross was maintained and observed for the presence or absence of the intersiphonal double band marker. The nomenclature used to identify each progeny colony was as follows: [maternal colony × paternal colony]-individual progeny identification number.

Crosses involving [Monterey × Woods Hole] $F_1$ colonies as parents were also set up in the manner described above.

## Results

### *Biology and morphology*

*Monterey Botryllus.* Colonies of Monterey *Botryllus* are usually found encrusting the surface of pilings, floats, rocks, seaweeds, and other sessile animals, such as *Ascidia ceratodes* (Tunicata) and *Phyllchaetopterus prolifica* (Annelidae), between lower intertidal and shallow subtidal zones in calm water. They compete with colonies of *Botrylloides violaceus* (belonging to the same family as *Botryllus*) in that habitat. In Monterey Marina, many sexually mature colonies are found from March to November; they are scarce in winter. Colonies sometimes grow to 7–8 cm across; the thickness is usually 1.0–1.5 mm. The colony surface is generally flat and free from any foreign matter. The tunic is soft, gelatinous, and transparent. When alive, colonies have orange, red-brown, or dark blue pigmentation. A colony (Fig. 1A) is composed of many zooids called blastozooids, which are arranged in oval or star-shaped systems in a common tunic. These systems are connected to one another, as are the zooids within any system itself, by a common vascular network in the tunic. Each system is composed of 5–15 blastozooids, which share a common cloacal aperture in the center of the system. The periphery of a colony is fringed with sausage-shaped vascular ampullae, each about 700 μm long and 200 μm wide, that project from the tunic's vascular array.

Each colony is founded by a sexually produced yellowish white or pale orange tadpole larva (Fig. 1B), about 1.6 mm long. The trunk of the larva is about 400 μm long, oval in outline, and has the single photolith typical of botryllids. Three adhesive papillae are arranged in a triangle on the anterior end of the trunk, and eight ampullae are arranged in a circular band surrounding the anterior half of the trunk. One or two hours after liberation the larvae attach to a suitable substratum by the adhesive papillae. Each larva extends its eight ampullae to complete attachment, and begins metamorphosis into a primary zooid (oozooid). The larva becomes a functional oozooid by opening its siphons and beginning to feed at 1.5–2 days after attachment.

An oozooid (Fig. 1C) is about 500 μm long and has four long transverse stigmata (protostigmata) on the left side, and four long ones, sometimes accompanied by a rudimental one, on the right side of the branchial sac. There is one inner longitudinal blood vessel on each side of the branchial sac. The stomach of an oozooid is 140 μm long and oval-shaped, with five longitudinal plications. The oozooid makes only one pallial bud, on the right side of the body. After one week, the feeding oozooid is resorbed by the colony and is replaced by its bud, which has developed into a functional zooid (the first blastogenic asexual generation). Buds of the next blastogenic generation develop on both sides of the first blastozooid. Replacement of zooids by new blastozooids of the next generation, called "takeover," occurs about once each week at 18–20°C and synchronously in all feeding zooids of a colony.

The first blastozooid (Fig. 1D, E) is 600–700 μm long and has four stigmatal rows on each side of the branchial sac; the second row does not reach the dorso-median line, and the fourth row is usually rudimentary. There is one inner longitudinal vessel on each side of the branchial sac. The stomach has five longitudinal plications. The first blastozooid usually produces a single bud on each side of the body; after the second blastogenic generation, blastozooids produce one or two buds on each side of their bodies.

The number of stigmatal rows in the blastozooids increases from one asexual generation to the next, ultimately resulting in eight rows. The number of inner longitudinal vessels on each side of the branchial sac also increases from one to three during the first several takeovers (Fig. 1F). Blastozooids of a mature colony (Fig. 2) lie obliquely in a common tunic and are 2.0–2.8 mm long. Branchial tentacles of a blastozooid consist of four large and four small ones, regularly alternating. There are usually eight rows of stigmata on each side of the zooids. The second row of stigmata never reaches the dorso-median line. Around the middle of the branchial sac, stigmata are arranged between the three inner longitudinal vessels as follows: dorsal lamina 5.2.3.4 endostyle (periods represent vessels; Fig. 2B). Many blood cells are deposited along each side of the endostyle in the range from the first to the sixth stigmatal row. The anterior edge of the intestinal loop reaches the level of the fifth or sixth transverse vessel, and the anus opens at the level of the sixth stigmatal row (Fig. 2B). Most of the stomach is exposed posterior to the branchial sac. The stomach is yellowish-orange or orange in fresh specimens, and is furnished with eight longitudinal plications and a long, hooked pyloric caecum.

The testis lies ventro-posterior to the ovary at the level of the 4th–5th stigmatal row on the left side, and at the level of the 6th or 7th stigmatal row on the right side (Fig. 2B, C). It consists of several white opalescent lobes forming a rosette. Eggs mature in the ovary of a bud during bud development and reach a maximum size of 230–250 μm just before takeover (Fig. 2C). Mature eggs are yellowish orange. They are ovulated when new blastozooids open their branchial siphons. The release of sperm occurs about two days after ovulation in the same zooids. Ovulation and sperm release occur synchro-

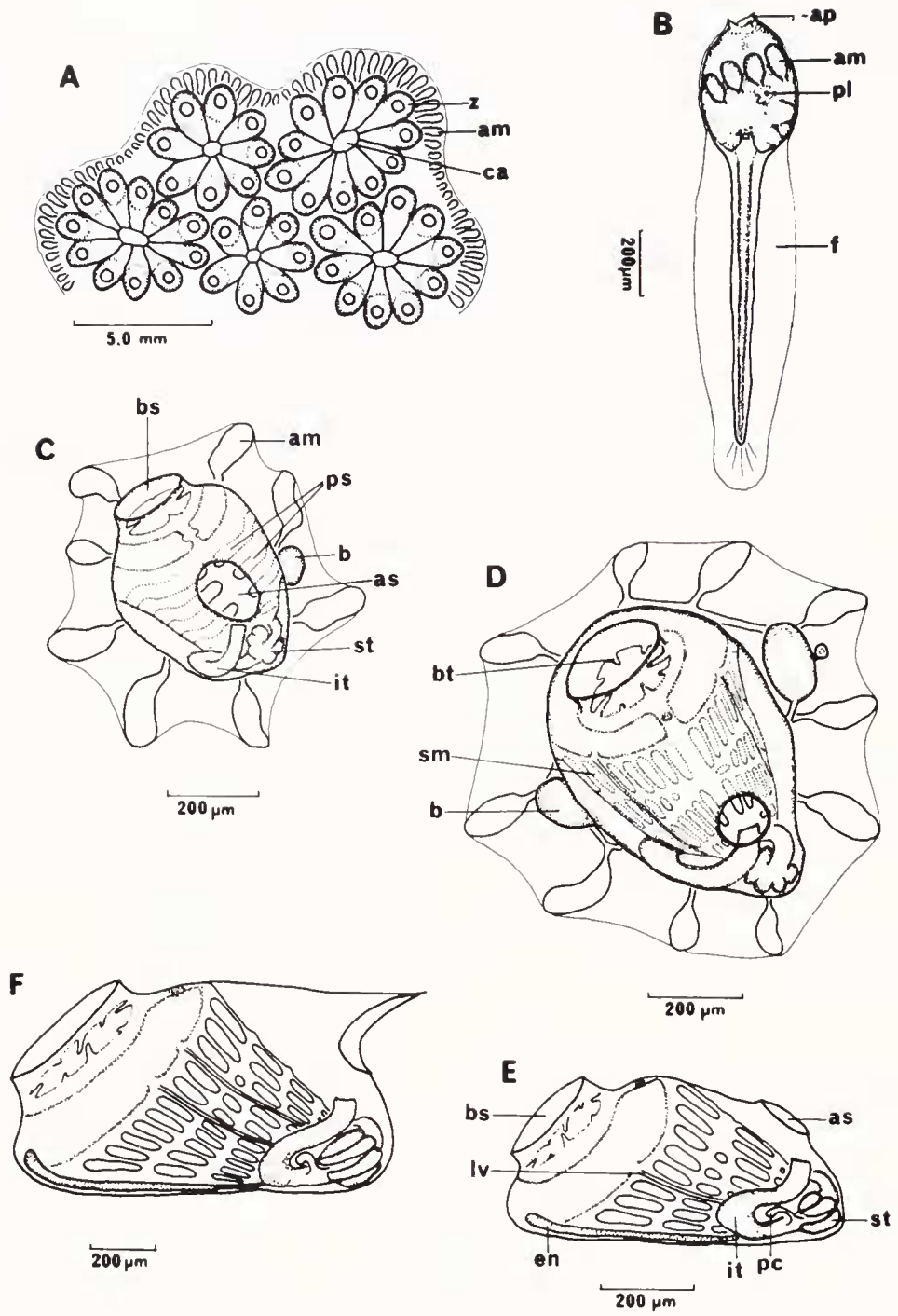


**Figure 1.** Monterey *Botryllus* (A) Part of a colony. Blastozooids are arranged into oval or star-shaped systems. (B) Larva, left side. (C) Oozooid, dorsal view. (D) First blastozooid, dorsal view, with buds of second blastogenic generation. (E) The same as (D), but left side. (F) Second blastozooid, left side. am, ampulla; ap, attachment process; as, atrial siphon; b, bud; bs, branchial siphon; bt, branchial tentacle; ca, cloacal aperture; en, endostyle; f, fin; it, intestine; lv, longitudinal vessel; pc, pyloric caecum; pl, photolith; ps, protostigmata; sm, stigmata; st, stomach; z, blastozooid.

nously in all zooids of a colony. The blastozooid of a healthy colony has one to three developing embryos supported by oviducal cups on each side of the peribranchial cavity (Fig. 2). The diameter of embryos just before hatching is about 280–300 μm.

*Woods Hole Botryllus.* We do not have year-round

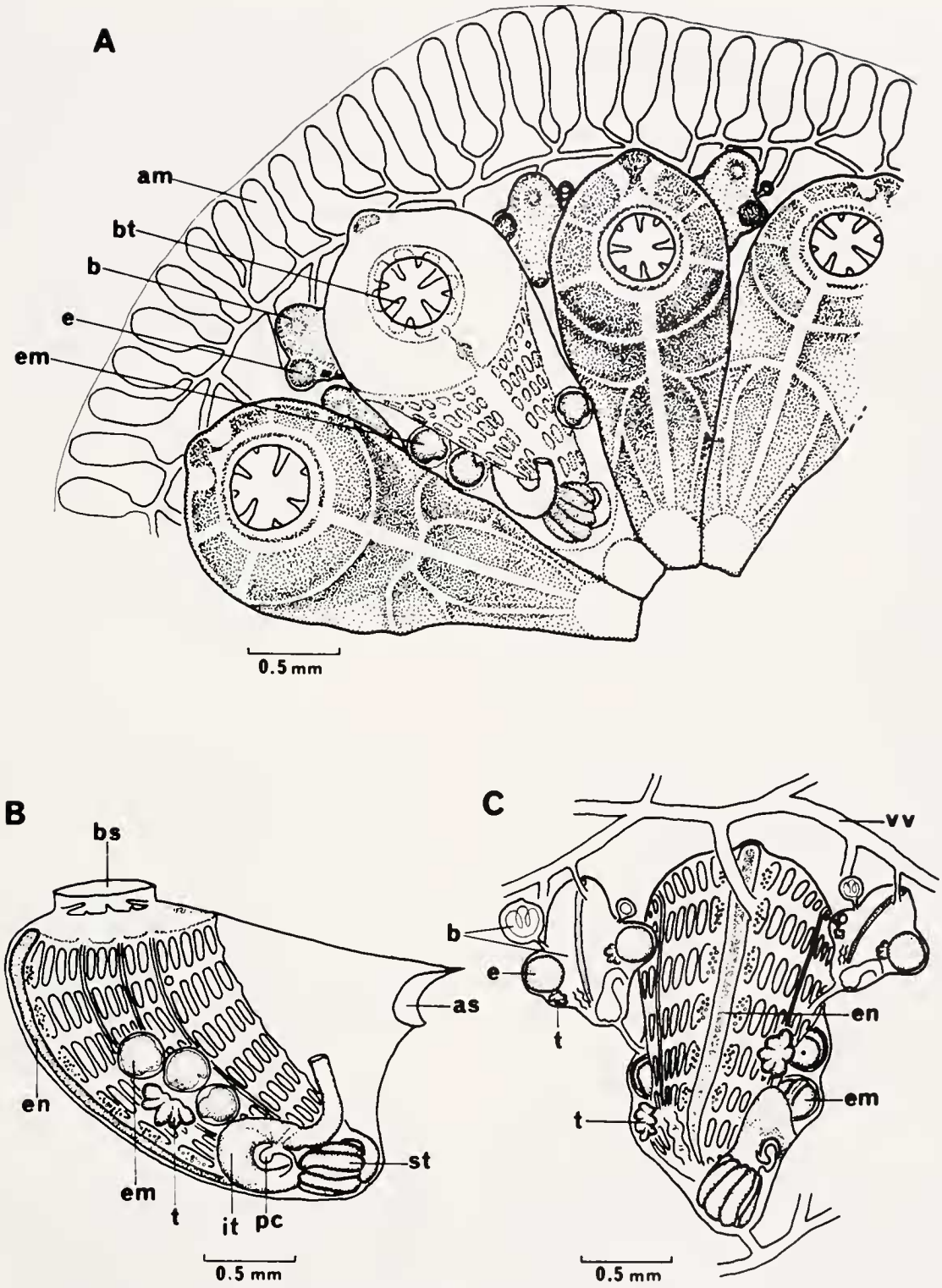


**Figure 2.** Monterey *Botryllus*. (A) Mature blastozooid, dorsal view. (B) The same as (A), but left side. (C) The same as (A), but ventral view with buds. am, ampulla; as, atrial siphon; b, bud; bs, branchial siphon; bt, branchial tentacle; e, egg; em, embryo; en, endostyle; it, intestine; pc, pyloric caecum; st, stomach; t, testis; vv, vascular vessel.

**Table I**

*Summary of morphology of Monterey and Woods Hole* Botryllus

| | Monterey[a] | Woods Hole[a] |
|---|---|---|
| *Colony* | | |
| Shape & thickness | Flat, 1.0–1.5 mm | Flat, 0.7–1.6 mm |
| Color & pigmentation | Orange, blue, brown; double intersiphonal band rarely present on zooids | Many variations; double intersiphonal band often present on zooids |
| Arrangement of zooid | Oval or star-shaped | Oval or star-shaped |
| Tunic (=test) | Soft, gelatinous, transparent | Soft, gelatinous, transparent |
| *Larva* | | |
| Total length | 1.6 mm | 1.5–1.7 mm |
| Trunk shape & size | oval, 400 μm long | oval, 400 μm long |
| Attachment processes | 3 | 3 |
| Ampullae | 8 | 8 |
| Photolith | 1 | 1 |
| *Oozooid* | | |
| Protostigmata | L:4, R:4 (+1) | L & R:4 |
| First ampullae | 8 | 8 |
| Longitudinal vessels | L & R:1 | L & R:1 |
| Stomach plications | 5 | 5 |
| First buds | R:1 | R:1 |
| *First blastozooid* | | |
| Sitgmatal rows | L & R:4 | L & R:4 |
| 2nd stigmatal row | Incomplete | Incomplete |
| Longitudinal vessels | L & R:1 | L & R:1 |
| Stomach plications | 5 | 5 |
| Number of buds | L:1, R:1 | L:1, R:1 |
| *Mature blastozooid* | | |
| Posture | Obliquely | Obliquely |
| Size | 2.0–2.8 mm long | 1.2–2.6 mm long |
| Branchial tentacles | 4 large & 4 small | 4 large & 2–4 small |
| Stigmatal rows | L & R:8 | L & R:7–8 |
| 2nd stigmatal row | Incomplete | Incomplete |
| Longitudinal vessels | L & R:3 | L & R:3 |
| Stomach plications | 8 | 7 |
| Pyloric caecum | Long, hooked | Long, hooked |
| Number of buds | L & R:1–2 | L & R:1 (lab-culture) |
| Testis shape & color | Rosette, white opalescent | Rosette, white opalescent |
| Embryos | L & R:1–3, supported with oviducal cups | L & R:1–3, supported with oviducal cups |
| Maximum size of embryos | 280–300 μm | 300 μm |
| Mature egg number | L & R:1–3 | L & R:1–3 |
| size in buds | 230–250 μm | 220–240 μm |
| Color of eggs | Yellowish orange | Yellow |

[a] L = left; R = right.

ecological data for Woods Hole *Botryllus*. However, we do have observations from a field study at the end of October 1986. According to these data, colonies of Woods Hole *Botryllus* were very common in the Eel Pond, but rare at the shore facing the open sea around the Marine Biological Laboratory. They were encrusting on the surfaces of pilings, floats, rocks, seaweeds, and solitary ascidians (*Styela partita*) in shallow water, and, like Monterey *Botryllus*, apparently were competing with colonies of *B. violaceus* for substrate. At that time, several of the colonies examined had developing embryos and eggs. Under laboratory culture conditions, Woods Hole colonies show the same life history as the Monterey colonies described above.

The detailed data in Table I (from observations on 30 to 100 colonies of each population) show that the morphology of Woods Hole *Botryllus* is similar to that of Monterey *Botryllus*, except for coloration. Therefore, only the few differences that exist between Woods Hole and Monterey colonies will be described here. The

Woods Hole population exhibited many color variations: orange, yellow, blue, green, brown, and mixtures of these colors. In many Woods Hole colonies, blastozooids had intersiphonal bands formed by deposition of white or yellow pigment cells between the branchial siphon and the atrial aperture (see Bancroft, 1903; Watterson, 1945; Sabbadin, 1962). This feature was very rare in Monterey colonies. Woods Hole colonies generally were thinner (0.7–1.6 mm) than Monterey colonies. Oozooids of Woods Hole *Botryllus* had four protostigmata on each side of the body; none (that we observed) had the rudimental fifth stigmata on the right side that was occasionally seen in Monterey colonies. Blastozooids of Woods Hole colonies (1.2–2.6 mm long) were somewhat smaller than those of Monterey colonies. In some adult Woods Hole colonies, blastozooids had four long branchial tentacles and only two small ones, and had seven stigmatal rows on each side of the branchial sac. There were seven stomach plications in Woods Hole colonies. The pattern of increase in the numbers of stigmatal rows, inner longitudinal vessels, and stomach plications during the first several takeovers, and the incompletion of the second stigmatal row, were the same for Woods Hole and Monterey colonies.

*Observations on colony specificity*

In colonial ascidians, when two colonies come into contact with each other, fusion or nonfusion occurs between them at the contact area. This allogeneic recognition phenomenon is called "colony specificity." Both fusion and nonfusion (allorejection) were observed in colony pairs from Monterey and in pairs from Woods Hole. Interpopulation pairs never fused, and the Woods Hole colony was always damaged by the Monterey colony. In these experiments, each different type of colony specificity reaction was observed in more than ten colony pairs, except for rejection in pairs from Woods Hole (five colony pairs). Within each colony pair, the assay was done in duplicate or triplicate.

The fusion process in both populations proceeded as follows. When two fusible colonies came into contact, vascular ampullae in the tunic of each colony extended toward those of the other colony (Fig. 3, Stage 1). Eventually, the tunic along the contact boundaries of the two colonies fused. The ampullae of each colony continued to grow out into the tunic of the facing colony (Fig. 3A, Stage 2), and finally their tips came into contact with the proximal (not distal) parts of ampullae of the other colony (Fig. 3A, Stage 3). At the contact points, the blood vessels of the two colonies became connected to one another; that is, a common vascular system was formed between the two colonies (Fig. 3A, Stage 4).

As with the fusible colonies, when two nonfusible colonies of the Monterey population came into contact with each other, the ampullae of each colony extended toward those of the other colony (Fig. 3, Stage 1). However, their tunics did not fuse, so the tips of ampullae of both colonies made contact with each other indirectly through the surface layers of their tunics (Fig. 3B, Stage 2). Bright green blood cells gathered near the tips of the ampullae at the contact areas, after which blood cells infiltrated the tunic around the ampullar tips (Fig. 3B, Stage 3). These blood cells changed from bright green to dark brown, and the ampullae of each colony withdrew from the contact areas (Fig. 3B, Stage 4).

When two nonfusible colonies from the Woods Hole population came into contact with each other, ampullae of each colony began to extend toward those of the other colony (Fig. 3, Stage 1). Fusion of their tunics occurred, but the area of tunic fusion was limited to a small space near the ampullar tips (Fig. 3C, Stages 2 and 3). The distal parts of the ampullae penetrating into the facing colony contracted and in some cases, were amputated, while blood cells from these ampullae infiltrated into the tunic (Fig. 3C, Stage 4).

When a Monterey colony and a Woods Hole colony were brought into contact with each other, their ampullae grew toward each other as described above for intraspecies pairs (Fig. 3, Stage 1). However, although the ampullar tips of both colonies came very close to one another, they did not make direct contact because tunic fusion did not occur (Fig. 3D, Stage 2). At the contact area, ampullae of the Woods Hole colony contracted and blood cells infiltrated the tunic from them, but the Monterey colony showed no such response (Fig. 3D, Stage 3). Ampullae of the Woods Hole colony were amputated or withdrew from the contact area, and the infiltrated blood cells in the tunic changed to a dark brown color (Fig. 3D, Stage 4).

*Production of $F_1$ progeny from crosses between Monterey and Woods Hole* Botryllus *colonies*

*Botryllus* colonies from Monterey and from Woods Hole are capable of both self- and cross-fertilization (Scofield *et al.*, 1982). The standard test of species identity requires that fertile $F_1$ hybrids be produced by cross-fertilization. In our study, two methods were used to demonstrate that cross-fertilization did occur between Monterey and Woods Hole colonies. First, because *Botryllus* colonies are protogynous (Milkman, 1967), eggs in a given colony are ready to be fertilized about two days before the autologous sperm are mature, and thus are fertilized preferentially by mature sperm from a heterologous colony if available (Sabbadin, 1962). Furthermore, the stages of a colony's asexual reproductive (*i.e.*, budding) cycle are interconnected in a predictable way

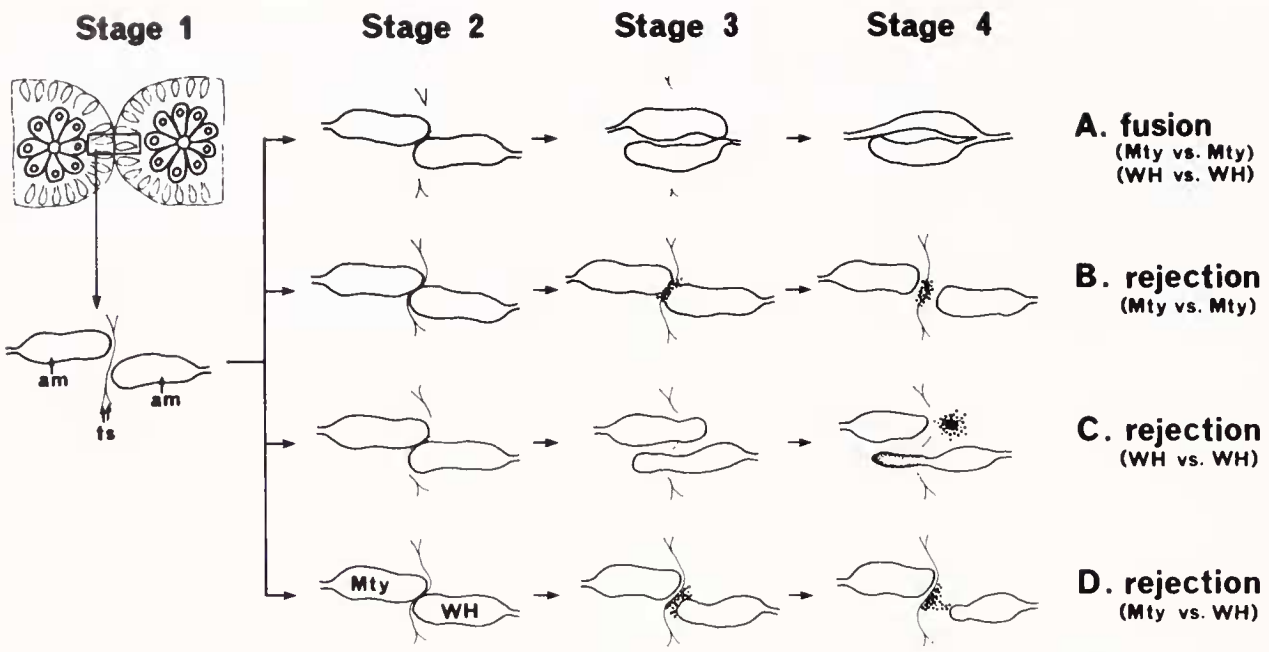


**Figure 3.** Scheme showing processes of fusion and rejection in Monterey and Woods Hole *Botryllus*. (A) Fusion between Monterey colonies and between Woods Hole colonies. (B) Rejection between Monterey colonies. (C) Rejection between Woods Hole colonies. (D) Rejection between Monterey and Woods Hole colonies. For detail, see text. am, ampulla; ts, tunic surface; Mty, Monterey colony; WH, Woods Hole colony.

with the developmental stages of its cross-fertilized embryos. Considering these traits in combination, we can dissect embryos from the maternal colony and determine whether they arose from self- or cross-fertilization. For example, if mature zooids that are about to undergo takeover (*i.e.*, each having a well-developed primary bud with a secondary bud attached to it via a stalk) contain late "wraparound" stage embryos, then these embryos must be the result of cross-fertilization (also see Milkman, 1967). On the other hand, if these zooids contain much earlier stage embryos, (*e.g.*, gastrulas), these embryos could be the result of self-fertilization. In the two crosses that were set up between Monterey and Woods Hole colonies, observations at selected times during embryonic development verified that reciprocal cross-fertilization had occurred between Monterey and Woods Hole colonies.

The second method for demonstrating that cross-fertilization had occurred depended on a genetically encoded pigmentation marker, the intersiphonal double band. This marker is controlled by a dominant allele at a single gene locus (Sabbadin and Graziani, 1967), and is relatively easy to see with a dissecting microscope (Fig. 4A). Colonies with double bands are more common in Woods Hole than in Monterey (pers. obs.). Therefore, we set up crosses in which one parent was a Monterey colony without double bands (Fig. 4B), and the other parent was a double-banded Woods Hole colony (Fig. 4A). In these cases we could be certain that any double-banded $F_1$ progeny that were released from the Monterey colony (*i.e.*, maternal colony lacking double bands) must have been derived from Monterey colony eggs fertilized by Woods Hole colony sperm bearing the allele for double bands.

The results of Monterey × Woods Hole crosses are shown in Table II. The numbers in this table are for samples of the progeny that were released. In both crosses, the Monterey and Woods Hole parental colonies each released at least 14 to 32 $F_1$ progeny, and among these $F_1$ progeny there were at least 9/32 (28%) to 9/14 (64%) double-banded colonies released from each of the four parents. These data indicate that crosses between Monterey and Woods Hole *Botryllus* colonies can produce viable $F_1$ offspring.

### *Monterey × Woods Hole $F_1$ progeny are fertile*

If *Botryllus* colonies from Monterey and Woods Hole belong to the same species, their $F_1$ progeny should be capable of sexual reproduction. An $F_1 \times F_1$ cross was set up between sibling colonies produced in the second cross in Table II. As shown in part (1) of Table III, both parental $F_1$ colonies, [Mty 248p.2 × WH 114] − 4 and [WH 114 × Mty 248p.2] − 3, released $F_2$ progeny. In this

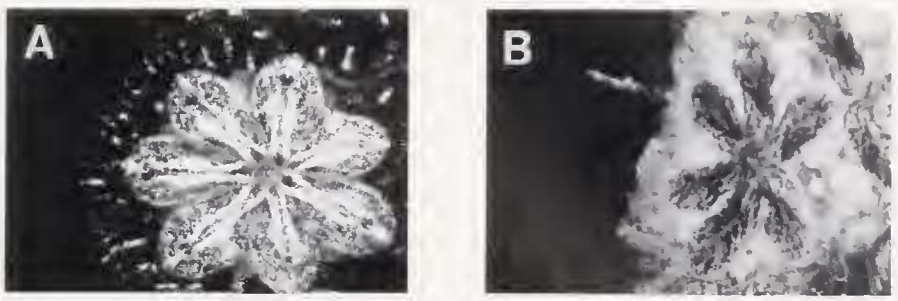


**Figure 4.** Representative portions of *Botryllus* colonies. (A) Woods Hole colony with intersiphonal double bands. (B) Monterey colony lacking intersiphonal double bands.

cross, the $F_1 - 4$ parent colony was a double-banded colony that was released from Mty 248p.2, which lacked the double band trait (Table II). Therefore, $F_1 - 4$ was definitely an $F_1$ hybrid resulting from cross-fertilization between Monterey and Woods Hole parental colonies. In this $F_1 \times F_1$ cross, the double band trait could not be used to distinguish between self- and cross-fertilization because both parents expressed it.

In the second cross (part 2, Table III), one parent, the double-banded [Mty 279u × WH 143] − 4 colony, was released from Mty 279u, which lacked the double band trait (Table II). Therefore, this $F_1 - 4$ colony was definitely an $F_1$ hybrid resulting from cross-fertilization between Monterey and Woods Hole parental colonies. The partner in this cross was a Monterey colony without double bands (Mty 405g). Both parental colonies released $F_2$ progeny, and at least 6 of 11 progeny released from Mty 405g were definitely the result of cross-fertilization because they expressed the double band trait obtained from the sperm donor $F_1 - 4$ (Table III).

In addition, as in the Monterey × Woods Hole crosses, dissections were performed at several time points during embryonic development in the crosses in Table III. We observed that the relationship between embryo stage and maternal colony asexual stage at given times was consistent with the expected schedule of cross-fertilization rather than self-fertilization. Thus, from this observation and the data in Table III, we conclude that Monterey and Woods Hole colonies can interbreed to produce fertile $F_1$ offspring, and therefore belong to the same *Botryllus* species.

**Table II**

*Results of crosses between Monterey and Woods Hole colonies*

| Pairs of parental colonies[a] | Double band in parent | Progeny[b,c] |
|---|---|---|
| (1) Mty 279u | − | 14 (9) |
| WH 143 | + | 29 (18) |
| (2) Mty 248p.2 | − | 32 (9) |
| WH 114 | + | 16 (6) |

[a] Mty = Monterey; WH = Woods Hole.

[b] The progeny on a given line in the table were released from the indicated colony when it was the egg donor (maternal colony) and the other colony of that pair was, therefore, the sperm donor.

[c] These are minimum numbers, because not all progeny that were released were collected and observed. The numbers in parentheses designate the minimum number of progeny, among those observed, that definitely showed the intersiphonal double band.

## Discussion

Four or more botryllid ascidians live along the coast of California (Fay and Johnson, 1971; Fay and Vallee, 1979; Abbott and Newberry, 1980), but only two of them have been identified: *Botryllus tuberatus* Ritter and Forsyth 1917 and *Botrylloides diegensis* Ritter and Forsyth 1917. The two unidentified species, one *Botryllus* and

**Table III**

*Results of crosses involving Monterey × Woods Hole $F_1$ progeny as parents*

| Pairs of parental colonies[a,b] | Double band in parent | Progeny[c,d] |
|---|---|---|
| (1) [Mty 248p.2 × WH 114] − 4 | + | 22 (11) |
| [WH 114 × Mty 248p.2] − 3 | + | 16 (9) |
| (2) [Mty 279u × WH 143] − 4 | + | 14 (6) |
| Mty 405g | − | 11 (6) |

[a] Mty = Monterey; WH = Woods Hole.

[b] The first three parental colonies in this table were $F_1$ progeny of Monterey × Woods Hole crosses. The nomenclature used to identify them is: [maternal colony × paternal colony] − individual progeny identification number.

[c] The progeny on a given line in the table were released from the indicated colony when it was the egg donor (maternal colony) and the other colony of that pair was, therefore, the sperm donor.

[d] These are minimum numbers, because not all progeny that were released were collected and observed. The numbers in parentheses designate the minimum number of progeny, among those observed, that definitely showed the intersiphonal double band.

one *Botrylloides*, are commonly found in Monterey Bay. From observations of its morphology and manner of sexual reproduction, we concluded that the latter is *Botrylloides violaceus* Oka 1927 described from Japan (Saito *et al.*, 1981b; Saito and Watanabe, 1985). The unidentified *Botryllus* has been considered to be very similar to *Botryllus schlosseri*, a worldwide species (Berrill, 1950), but Abbott and Newberry (1980) hesitated to call it *B. schlosseri*. Here we have compared the morphology and some other biological characteristics of Monterey *Botryllus* and Woods Hole *Botryllus* (the latter considered to be *B. schlosseri*) in detail, and examined their colony specificity. In addition, we have set up crosses between colonies from these two populations.

As shown in Table I, the morphological characteristics of individuals from both Monterey and Woods Hole populations throughout their life cycles were very similar, except for coloration. Colonies of Monterey *Botryllus* were generally orange, red-brown, or blue, whereas Woods Hole colonies have many color variations, and many colonies have intersiphonal pigment bands. However, differences in colony color or pigmentation markers usually are not of much taxonomic significance in ascidians (Van Name, 1945). In colonies from both populations, blastozooids are arranged into oval or star-shaped systems; this characteristic is seen in *B. schlosseri*, *B. tuberatus*, *Botryllus primigenus* Oka 1928, and *Botryllus communis* Oka 1927.

The number of longitudinal vessels on each side of the branchial sac increases from one to three during the first several blastogenic generations in colonies from both populations. This fact has never been reported in either *B. schlosseri* or other botryllid ascidians, but if detailed observations are carried out in other botryllids the same increase will be found in many of them (pers. obs.). The second stigmatal row never reaches the dorso-median line in either Monterey or Woods Hole *Botryllus*, and this characteristic has already been reported in Japanese *Botryllus* and *Botrylloides* (Tokioka, 1953; Saito *et al.*, 1981a, b; Saito and Watanabe, 1985). This seems to be a common characteristic in botryllids with more than five stigmatal rows. There are some minor differences between Monterey and Woods Hole *Botryllus:* colony thickness, blastozooid length, and numbers of branchial tentacles, of stigmatal rows, and of stomach plications. However, these are differences that plausibly fall within the range of normal intraspecies variation between widely separated populations.

In addition to morphological similarity, members of a species can interbreed to produce fertile offspring (Friday and Ingram, 1985). We developed controlled culture conditions for raising Monterey *Botryllus* colonies from larval release to sexual maturity in the laboratory (Boyd *et al.*, 1986), and these conditions were also suitable for maintaining Woods Hole colonies. Successful laboratory culturing, along with the fact that *Botryllus* colonies reach sexual maturity within a few months and subsequently can breed frequently, permitted us to answer directly whether crosses yield fertile $F_1$ offspring. As indicated by our data (Tables II, III), Monterey and Woods Hole colonies can interbreed to produce fertile $F_1$ progeny. The second cross in Table III provides definitive evidence that *cross*-fertilization occurred with the [Monterey $\times$ Woods Hole] $F_1$ colony. The results of the defined crosses indicate that Monterey and Woods Hole *Botryllus* colonies belong to the same species.

The manner of allorejection in colony specificity has generally been considered to be consistent between colonies of the same species of botryllid (Taneda *et al.*, 1985). However, allorejection between Monterey colonies was distinctly different from that between Woods Hole colonies. Moreover, in interpopulation pairs, only the Woods Hole colony consistently exhibited a rejection reaction. That fusion was not observed between Monterey and Woods Hole colonies in this study is probably related to the frequency and distribution of alleles at the fusibility locus within the two *Botryllus* populations, such that none of the interpopulation pairs happened to include colonies that shared an allele at this locus. Based on the similarity of their morphology and the production of fertile $F_1$ progeny, it is best to consider that they belong to the same species, although they may be divergent with respect to the allorecognition process. Even though allorejection has never been used as a taxonomic characteristic, this reaction could prove useful for future studies of botryllid taxonomy.

Monterey and Woods Hole *Botryllus* appear to be in the same species, but the question remains whether they are *B. schlosseri*. Although *B. schlosseri* has been called a worldwide species (Van Name, 1945), there seem to be some inconsistencies among reports about this species and our data on Monterey and Woods Hole *Botryllus*. (1) According to Colombera (1969), the haploid number of chromosomes in Italian colonies is 16, whereas it has been reported to be 7 or 8 in Woods Hole colonies (Milkman and Therrien, 1965). (2) In the description by Berrill (1950), an oozooid has eight to nine protostigmata on the right side and six to seven on the left side. However, in Monterey and Woods Hole populations an oozooid usually has four protostigmata on each side. (3) In the same description by Berrill, an oozooid produces one pallial bud of the first blastogenic generation on each side of the body, but in Monterey, Woods Hole, and Italian populations, an oozooid produces a single bud only on the right side of the body (for Italy, see Brunetti and Burighel, 1969; Sabbadin, 1969, 1979). (4) The diameter of mature eggs is about 450 $\mu$m in Berrill's (1950) description and 410–430 $\mu$m in Korean colonies (Rho, 1971). In

Monterey and Woods Hole colonies, and in some other descriptions (Ärnbäck, 1923; Van Name, 1945; Brewin, 1946), the diameter is 220–265 μm. (5) There is much variation in the eventual number of stigmatal rows, from 6 to 15, among descriptions of *B. schlosseri* (Savigny, 1816; Verrill, 1871; Alder and Hancock, 1912; Michaelsen, 1921; Hartmeyer, 1923; Van Name, 1945; Brewin, 1946; Berrill, 1950; Tokioka, 1953, 1967; Rho, 1971; Kott, 1972; Kott and Goodbody, 1980; Millar, 1982; Ger and Zan, 1983).

These inconsistencies in the basic characteristics of botryllid ascidians suggest that more than two species are currently classified as *B. schlosseri*. In order to clarify this problem, colonies from populations around the world must be used (1) in studies of colony specificity and (2) of zooid morphology throughout the life cycle and (3) for defined crosses to determine whether fertile $F_1$ progeny can be produced. This taxonomic study would be very important for the several fields of biology using this species for experimental and ecological investigations. In the meantime, researchers should be cautious about applying information and conclusions from experiments on one *Botryllus* population to colonies belonging to other populations. Our present understanding of the taxonomy and genetics of *B. schlosseri* leads us to designate Monterey and Woods Hole colonies as conspecific, but it is premature to definitively call that species *Botryllus schlosseri*. Normally that identification must await comparison of Monterey or Woods Hole specimens with the type-specimen that actually carries the taxon's name—a task that has not yet been undertaken. If that is not feasible, other studies comparable to this one can, at least, ascertain whether the Monterey-Woods Hole taxon is conspecific with populations from *B. schlosseri*'s European type-location (Falmouth, England), and then with other important European populations (*e.g.*, Venice). In fact, this sort of extended comparison would be even preferable, because it permits the live animal breeding experiments that would not be possible in an analysis solely dependent on type-specimens.

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